ANTIMICROBIAL PEPTIDES: PORE FORMERS OR METABOLIC INHIBITORS IN BACTERIA?

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Abstract | Antimicrobial peptides are an abundant and diverse group of molecules that are produced by many tissues and cell types in a variety of invertebrate, plant and animal species. Their amino acid composition, amphipathicity, cationic charge and size allow them to attach to and insert into membrane bilayers to form pores by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms. Although these models are helpful for defining mechanisms of antimicrobial peptide activity, their relevance to how peptides damage and kill microorganisms still need to be clarified. Recently, there has been speculation that transmembrane pore formation is not the only mechanism of microbial killing. In fact several observations suggest that translocated peptides can alter cytoplasmic membrane septum formation, inhibit cell-wall synthesis, inhibit nucleic-acid synthesis, inhibit protein synthesis or inhibit enzymatic activity. In this review the different models of antimicrobial-peptide-induced pore formation and cell killing are presented.

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The antimicrobial activities of secretions, blood, leukocytes, and lymphatic tissues were recognized as early as the "last fifteen years of the nineteenth century"[1], and between 1920 and 1950 many antimicrobial compounds that were isolated from these secretions were shown to be selective for Gram-positive and Gram-negative bacteria[1]. The list of compounds included a bacteriolytic substance in nasal mucous (which was later named lysozyme[2]), basic antimicrobial proteins and basic linear tissue polypeptides. Although some of the larger basic proteins were thought to be histone fractions and protamine, the identity of the small tissue polypeptides was unknown. Despite this, the descriptions of their characteristics, activities and modes of action were accurate: "…antimicrobial basic proteins and polypeptides combine with cell nucleoproteins or other negatively charged surface constituents of bacteria or viruses, thus disrupting important cell function. The union of the basic substances with negatively charged cell surfaces is believed to occur through electrostatic bonding"[1]. The association of the presence of these antimicrobial substances in normal tissues and fluids with natural resistance to microorganisms was clearly made. They were described as being inducible on exposure to infecting microorganisms, to kill or slow the growth of invading microorganisms and to aid allied mechanisms of natural and adaptive immunity. Thus the field of antimicrobial peptide research was born. Shortly afterwards, antimicrobial substances were purified from phagocytic granule extracts by Hirsch[3] and correlated with the presence of low-molecular-mass cationic compounds in granule mixtures[4–6], including bactericidal/permeability-increasing protein[7]. The field expanded further when Hans Boman, Michael Zasloff and Robert Lehrer independently isolated and purified insect cecropins, amphibian magainins and mammalian defensins, respectively[8–10]. Now, more than 880 different antimicrobial peptides have been identified or predicted from nucleic acid sequences (see Anti-infective peptides in the Online links box). These include antimicrobial peptides that are produced in many tissues and cell types of a variety of invertebrate, plant and animal species[11–15], certain cytokines and chemokines[16–18], selected neuropeptides and peptide hormones[19,20], and fragments of

Box 1 | **Classes of antimicrobial peptides**

Anionic peptides
- Maximin H5 from amphibians[146].
- Small anionic peptides rich in glutamic and aspartic acids from sheep, cattle and humans[30].
- Dermcidin from humans[147].

Linear cationic α-helical peptides
- Cecropins (A), andropin, moricin, ceratotoxin and melittin from insects.
- Cecropin P1 from *Ascaris* nematodes[148].
- Magainin (2), dermaseptin, bombinin, brevinin-1, esculentins and buforin II from amphibians.
- Pleurocidin from skin mucous secretions of the winter flounder.
- Seminalplasmin, BMAP, SMAP (SMAP29, ovispirin), PMAP from cattle, sheep and pigs.
- CAP18 from rabbits.
- LL37 from humans.

Cationic peptides enriched for specific amino acids
- Proline-containing peptides include abaecin from honeybees[28].
- Proline- and arginine-containing peptides include apidaecins from honeybees[28]; drosocin from *Drosophila*[28]; pyrrhocoricin from the European sap-sucking bug[36]; bactenecins from cattle (Bac7), sheep, and goats[149]; and PR-39 from pigs[73,150].
- Proline- and phenylalanine-containing peptides include prophenin from pigs[150].
- Glycine-containing peptides include hymenoptaecin from honeybees[28].
- Glycine- and proline-containing peptides include coleoptericin and holotricin from beetles[28].
- Tryptophan-containing peptides include indolicidin from cattle[151].
- Small histidine-rich salivary polypeptides, including the histatins from man and some higher primates[116].

Anionic and cationic peptides that contain cysteine and form disulphide bonds
- Peptides with 1 disulphide bond include brevinins[152].
- Peptides with 2 disulphide bonds include protegrin from pigs and tachyplesins from horseshoe crabs[153].
- Peptides with 3 disulphide bonds include α-defensins from humans (HNP-1, HNP-2, cryptidins), rabbits (NP-1) and rats[154]; β-defensins from humans (HBD1, DEFB118), cattle, mice, rats, pigs, goats and poultry[12]; and rhesus θ-defensin (RTD-1) from the rhesus monkey[40].
- Insect defensins (defensin A)[95].
- SPAG11/isoform HE2C, an atypical anionic β-defensin[42].
- Peptides with >3 disulphide bonds include drosomycin in fruit flies[155] and plant antifungal defensins[155].

Anionic and cationic peptide fragments of larger proteins
- Lactoferricin from lactoferrin.
- Casocidin I from human casein.
- Antimicrobial domains from bovine α-lactalbumin, human haemoglobin, lysozyme and ovalbumin.

larger proteins[21–23]. In fact, the list of host-derived antimicrobial molecules is increasing so rapidly that one has to question the biological relevance and likely roles in innate immunity, particularly some antimicrobial fragments of larger proteins.

Antimicrobial peptides are recognized as a possible source of pharmaceuticals for the treatment of antibiotic-resistant bacterial infections or septic shock[24,25]. Studies to assess the mechanisms of natural peptide and peptide congener activity in model membrane systems, combined with similar studies of the same peptides with microorganisms, have helped to identify the parameters that are required for optimal peptide activity. In this review, the different models of antimicrobial peptide activity are presented with a discussion on the relevance of the mechanisms to antimicrobial-peptide-induced killing of microorganisms. Antimicrobial peptides can also inactivate nucleic acids and cytoplasmic proteins, and evidence of this as a mechanism for antimicrobial-peptide-induced killing of microorganisms is included.

Antimicrobial peptide diversity

Antimicrobial peptides are a unique and diverse group of molecules (BOXES 1,2), which are divided into subgroups on the basis of their amino acid composition and structure[15,26–28]. The NMR solution structures of selected peptides of these subgroups are shown in FIG. 1. One subgroup contains anionic antimicrobial peptides. Among these are small (721.6–823.8 Da) peptides present in surfactant extracts, bronchoalveolar lavage fluid and airway epithelial cells[29–31]. They are produced in mM concentrations, require zinc as a cofactor for antimicrobial activity and are active against both Gram-positive and Gram-negative bacteria. They are similar to the charge-neutralizing pro-peptides of larger zymogens, which also have antimicrobial activity when synthesized alone[32].

A second subgroup contains ~290 cationic peptides, which are short (contain <40 amino acid residues), lack cysteine residues and sometimes have a hinge or 'kink' in the middle[26,33] (see Anti-infective peptides in the Online links box). In aqueous solutions many of these peptides are disordered, but in the presence of trifluoroethanol, sodium dodecyl sulphate (SDS) micelles, phospholipid vesicles and liposomes, or Lipid A, all or part of the molecule is converted to an α-helix[26]. A good example is LL-37. In water, it exhibits a circular dichroism (CD) spectrum that is consistent with a disordered structure[34]. However, in 15 mM HCO_3^-, SO_4^{2-} or $CF_3CO_2^-$, the peptide adopts a helical structure. As has been observed for buforin II, its congeners and LL-37, the extent of α-helicity correlates with the antibacterial activity against both Gram-positive and Gram-negative bacteria — increased α-helical content correlates with stronger antimicrobial activities[35].

A third subgroup contains ~44 cationic peptides that are rich in certain amino acids[36] (see Anti-infective peptides in the Online links box). This group includes the bactenecins and PR-39, which are rich in proline (33–49%) and arginine (13–33%) residues; prophenin, which is rich in proline (57%) and phenylalanine (19%) residues; and indolicidin, which is rich in tryptophan residues[26,36]. These peptides lack cysteine residues and are linear, although some can form extended coils.

A fourth subgroup of anionic and cationic peptides have ~380 members, contain cysteine residues and form disulphide bonds and stable β-sheets (see Anti-infective peptides in the Online links box). This subgroup includes protegrin from porcine leukocytes (which comprises 16 amino acid residues, including

Box 2 | **Characteristics that affect antimicrobial activity and specificity**

Size
The size of antimicrobial peptides varies from 6 amino acid residues for anionic peptides to greater than 59 amino acid residues for Bac7. Even di- and tripeptides with antimicrobial activity have been reported.

Sequence
Peptides often contain the basic amino acid residues lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan, and other residues such as isoleucine, tyrosine and valine. Some peptides contain amino acid repeats. Ratios of hydrophobic to charged residues can vary from 1:1 to 2:1.

Charge
Anionic peptides are rich in aspartic and glutamic acids and cationic peptides are rich in arginine and lysine. Anionic peptides that are complexed with zinc, or highly cationic peptides, are often more active than neutral peptides or those with a lower charge.

Conformation and structure
Antimicrobial peptides can assume a variety of secondary structures including α-helices, relaxed coils and antiparallel β-sheet structures. Amphipathic α-helical peptides are often more active than peptides with less-defined secondary structures. Peptides with a γ-core motif (two antiparallel β-sheets with an interposed short turn in defensin-like molecules) are often very active.

Hydrophobicity
This characteristic enables water-soluble antimicrobial peptides to partition into the membrane lipid bilayer.

Amphipathicity
A trait by which peptides contain hydrophilic amino acid residues aligned along one side and hydrophobic amino acid residues aligned along the opposite side of a helical molecule. For α-helical peptides, amphipathicity is often expressed as a hydrophobic moment, which is the vector sum of hydrophobicity indices, treated as vectors normal to the helical axis. Other peptides often show spatial separation of polar and hydrophobic residues that is less easy to quantify.

four cysteines that are linked by two intramolecular disulphide bonds), and a diverse family of defensins. There are ~55 α-defensins, which include human neutrophil peptides (HNPs) and cryptdins and comprise 29–35 amino acid residues, including six cysteines that are linked by three intramolecular disulphide bonds[37]. There are ~90 β-defensins from both humans (HBDs) and animals that comprise 36–42 amino acid residues including six cysteines that are linked by three intramolecular disulphide bonds[9,38,39]. In addition, there are ~54 arthropod (insect) defensins, ~58 plant defensins and a rhesus θ-defensin (RTD-1), which is an 18-residue peptide that forms a circular molecule that is crosslinked by three disulphide bonds[40,41]. SPAG11/isoform HE2C is an atypical anionic β-defensin-like peptide[42].

Finally, there are anionic and cationic peptides that are fragments of larger proteins. These fragments have antimicrobial activity and are similar in composition and structure to the antimicrobial peptides described above. However, their role in innate immunity is not yet clear.

Mechanisms of antimicrobial peptide activity

A variety of techniques have been used to assess the mechanisms of antimicrobial peptide activity. However, each method (briefly described below) provides a slightly different view of peptide activity and no single technique is capable of adequately determining the mechanism of action of the peptides.

Microscopy. The use of microscopy to visualize the effects of antimicrobial peptides on microbial cells has helped to identify general target sites. Confocal laser-scanning microscopy has shown that biotinylated magainin 2 binds to the cell surface, whereas biotinylated buforin II enters the cell and accumulates in the bacterial cytoplasm[35]. The internal proline hinge in buforin II is important for peptide penetration and analogues that lack this hinge only localize to the cell surface. Scanning and transmission electron microscopy have been used to demonstrate the damaging effects of antimicrobial peptides such as SMAP29 on the ultrastructure of microbial cells (FIG. 2). Microscopic analyses have shown that different antimicrobial peptides have different effects on microbial cells, which indicates that different peptides have different target sites or mechanisms of activity — as has been shown for the effects of SMAP29 and CAP18 on *Pseudomonas aeruginosa*[43]. In many instances, cellular damage lags substantially behind the time required for antimicrobial killing, indicating that some of the ultrastructural damage is artefactual. In other instances, cellular damage occurs at the same rate as that of killing. Lehrer *et al.* noted that membranous blebs on HNP-treated *Escherichia coli* continued to accumulate as viable counts decreased and concluded that the appearance of blebs followed, rather than caused, the loss of bacterial viability[44]. In similar studies, *E. coli* incubated with DEFB118 were killed in 15 minutes but cellular damage continued 30–120 minutes after exposure to the peptide[45]. Finally, *P. aeruginosa* cells incubated with SMAP29 or CAP18 were killed in 15 minutes, whereas cellular damage continued for up to 8 hours[43], and SMAP29 and CAP18 were detected in the cytoplasm by immunoelectron microscopy almost immediately after the cells were exposed to peptide.

Studies with model membranes. Assessing the interaction of antimicrobial peptides with phospholipids in model membranes to provide insights into mechanisms of activity might be more relevant than using electron microscopy to determine the type of cellular damage induced by peptides. Single or mixed lipids are prepared as membranes or vesicles and incubated with antimicrobial peptides. The attraction, attachment, insertion and orientation of the peptide, as well as the orientation of the lipids and the thickness and integrity of the lipid bilayer can be measured by X-ray crystallography, NMR spectroscopy (both of peptides in solution and in the presence of lipids bilayers), and Fourier transform infrared (FTIR), Raman, fluorescence or CD optical spectroscopy. The relevance of these methods to antimicrobial peptide activity against microorganisms varies with the technique. These methods do, however, show that there are definite peptide composition and activity relationships that could be relevant to the design and synthesis of future antimicrobial peptide pharmaceuticals.

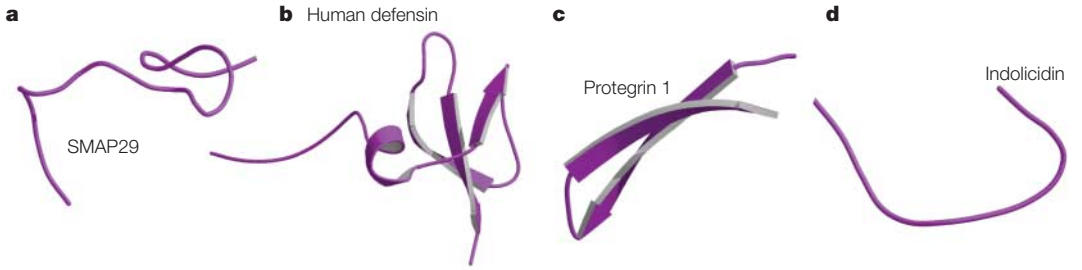

Figure 1 | **Antimicrobial peptides.** NMR solution structures and amino acid sequences are shown. **a** | SMAP29 (PDB code 1fry; RGLRRLGRKIAHGVKKYGPTVLRIIRIAG). **b** | Human β-defensin 3 (PDB code 1kj6; DHYNCVSSGGQCLYSACPIFTKIQGTCYRGK-AKCCK). **c** | Protegrin 1 (PDB code 1pg1; RGGRLCYCRRRFCVCVGR). **d** | Indolicidin (PDB code 1qxq; ILPWKWPWWPWRRG). These structures are representative of linear α-helical peptides (**a**), peptides enriched for specific amino acids (**d**) and peptides containing cysteine residues that form disulphide bonds (**b**,**c**). NMR solution structures of indolicidin bound to dodecylphosphocholine micelles (PDB code 1g89) or sodium dodecyl sulphate micelles (PDB code 1g89) are available in PDBsum but are not shown here. High-resolution images of peptide backbones were obtained from PDBsum and generated using Molscript[160] and Raster3D[161], with permission from Roman Laskowski, European Bioinformatics Institute, Wellcome Trust Genome Campus, Cambridge, United Kingdom.

Fluorescent dyes. The ability of cecropin A, magainin 2, indolicidin, melittin, rabbit neutrophil defensins and other antimicrobial peptides to permeabilize membrane vesicles can be measured by the release of internal fluorescent-labelled dextran, immunoglobulins, calcein or other probes[46–50]. The effects of time, peptide concentration and membrane composition on labelled probe release can be measured[51]. Melittin, for example, induces the formation of 2.5–3.0-nm diameter pores in palmitoyloleoylphosphatidylcholine vesicles at a lipid/peptide ratio of 50 (REF. 46), and the diameter of the pores generally increases with the lipid/peptide ratio[47]. Defensins can also permeabilize vesicles containing lipid mixtures that mimic the composition of bacterial membranes[49].

Ion channel formation. Monitoring voltage-dependent channels in membrane bilayers is another useful technique for assessing the formation and stability of an antimicrobial-peptide-induced pore. The ability of antimicrobial peptides to attach to and penetrate the bilayer is measured by the conductivity of an electrical current generated through the subsequently formed pore. With this technique, 0.1–10 μM of cecropin was shown to form large, time-variant and voltage-dependent 4-nm ion channels[52]. This estimate correlates well with the small lesions (9.6-nm pores with an inside diameter of 4.2 nm) that were observed using electron microscopy in cecropin-treated *E. coli*[53]. Some defensins also permeabilize planar lipid bilayer membranes at concentrations that are almost identical to those required to kill cells *in vitro*, showing that, in this model, the physiological effects of HNP-1 and NP-1 were relevant to their cytotoxic mechanisms[54].

Circular dichroism and orientated circular dichroism. The orientation and secondary structure of an antimicrobial peptide bound to a lipid bilayer can be measured by CD in a controlled humidity environment with light incident normal to the sample surface[55,56]. Using CD, indolicidin assumes a disordered conformation in aqueous and bulk organic solutions, and an ordered, but not α-helical, conformation in SDS micelles and lipid bilayers[57]; tachyplesin I, which is not AMPHIPATHIC in a buffer containing 20 mM Tris-HCl, 100 mM NaCl (pH 8.0), becomes amphipathic in the presence of phosphatidylcholine liposomes[58]; LL-37 is converted from a random coil to an α-helix in the presence of solvents[34] or Lipid A[59]; and in solvents dermaseptin b adopts an amphipathic α-helical conformation that most closely resembles those of class L amphipathic helices, in which all the lysine residues are located on the polar face of the helix[60]. These examples show that the local environment at the bacterial outer surface and membranes is important and can induce antimicrobial peptide conformational changes that are necessary for peptide attachment to and insertion into the membrane.

Solid-state NMR spectroscopy. Solid-state NMR spectroscopy measures the secondary structure, orientation and penetration of antimicrobial peptides into lipid bilayers in the biologically relevant LIQUID-CRYSTALLINE STATE[61,62]. These data help to define the interactions of antimicrobial peptides with bacterial membranes and the effects of peptide and membrane composition on activity. Magainin[63], ovispirin[64] and LL-37 (REF. 65) are positioned parallel to the plane of the lipid bilayer and protegrin was found to lie ~55° tilted with respect to the bilayer surface[62]. The hydrophobic backbone of protegrin interacts with the hydrophobic core of the bilayer, which allows the cationic arginine side chains to interact with the anionic phosphate groups. RTD-1 resembles protegrin in both primary and secondary structures; however, in protegrin, the arginine residues are located at the ends of the β-hairpin making it an amphipathic molecule, whereas in RTD-1 they are located throughout the molecule[66]. RTD-1 binds asymmetrically to bilayer phospholipid head groups on the outer leaflet. This induces a weak curvature in the membrane, which results in the formation of a membrane cylinder[66]. The relevance of this finding for the role of peptides in innate immunity is not clear.

AMPHIPATHIC
Here, used to describe peptides containing both hydrophilic and hydrophobic amino acid residues, where spatial separation of these residues facilitates their attachment and insertion into membranes.

LIQUID-CRYSTALLINE STATE
The state and temperature at which hydrocarbon tails of the lipid bilayers are fluid and can move. In most biomembranes, the lipids are in the liquid-crystalline state under physiological conditions.

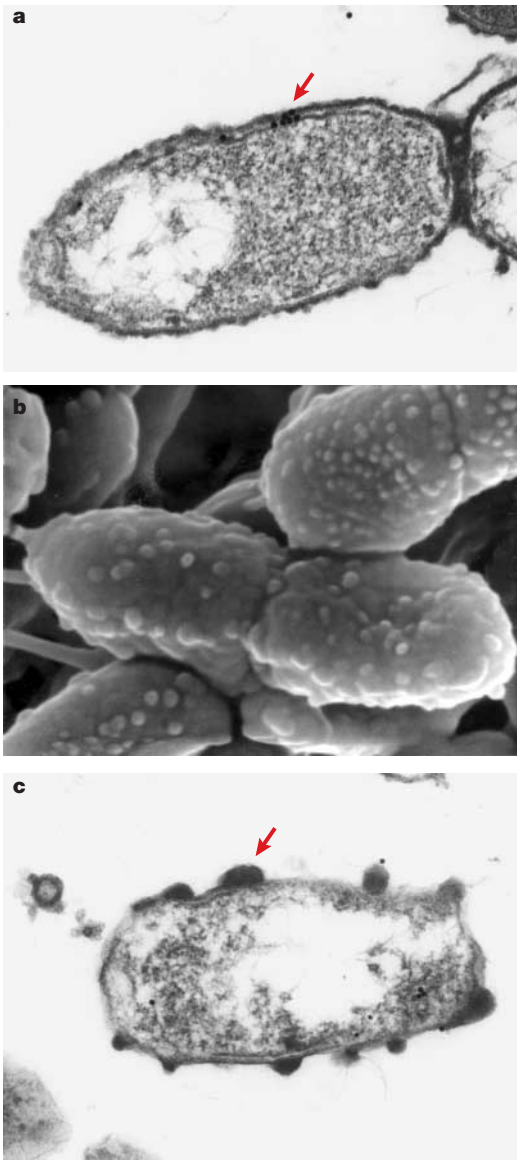

Figure 2 | ***Pseudomonas aeruginosa*** **PAO1 (10^8 cfu ml^{-1}) incubated with the ovine cathelicidin SMAP29 (10 μg ml^{-1}) for 1.5 and 3 hours. a** | Specific antibody and protein A colloidal gold labelling show SMAP29 attached to the outer leaflet of the cytoplasmic membrane (arrow). **b** | In other cells, SMAP29-induced blebs start to form and protrude from the microbial surface. **c** | By 3 hours, the blebs continue to grow and, in cross section, appear as electron-dense material attached to the microbial surface (arrow). Panel **b** courtesy of Hong Peng Jia and Paul McCray Jr, Department of Pediatrics, University of Iowa College of Medicine, USA.

NEUTRON IN-PLANE SCATTERING
A neutron-diffraction pattern of a peptide and membrane sample with the multilayer sample oriented normal to the incident neutron beam.

NEUTRON OFF-PLANE SCATTERING
A neutron-diffraction pattern of a peptide and membrane sample in a sandwiched multilayer sample oriented at an oblique angle with respect to the incident neutron beam, so that the entire low-angle diffraction pattern can be recorded by the area detector at one sample-to-detector distance.

Neutron diffraction. Alamethicin is a 20-amino-acid peptide that is produced by the fungus *Trichoderma viride*[67]. NEUTRON IN-PLANE SCATTERING detects alamethicin- and magainin-induced pores in membranes in which the neutron scattering-length densities are different from those of the membranes without alamethicin or magainin[68,69]. NEUTRON OFF-PLANE SCATTERING is a simple and efficient method of recording the diffraction patterns of peptide-induced pores within membranes in oriented multilayers[70] or liquids[71]. The use of deuterium provides contrast, highlighting the water-filled pores in the bilayers against a lipid background[71]. By examining the contrast variations, the inner and outer pore diameters can be accurately measured[71]. The in-plane scattering curves of alamethicin and magainin are similar, but the off-plane scattering patterns are markedly distinct[70], indicating that, although they both form pores in membranes, the pores are of different sizes[71].

Other techniques. Lamellar X-ray diffraction has shown that antimicrobial peptides cause concentration-dependent membrane thinning[55,72]. Synchrotron-based X-ray scattering has been used to determine the conformation of alamethicin in highly aligned stacks of model lipid membranes, and differences in scattering signal were detected between samples of high molar peptide/lipid ratio and samples of an ideal helix in the transmembrane state[67].

Antimicrobial-peptide-mediated cell killing

Peptide-mediated cell killing can be rapid. Some linear α-helical peptides kill bacteria so quickly that Boman reported that it is technically challenging to characterize the steps (if there are any) preceding cell death[28]. Other peptides, such as magainin 2 (REF. 10), cecropin P1 (REF. 73), PR-39 (REF. 73) and SMAP29 (REF. 43), kill bacteria in 15–90 minutes. Regardless of the time required, or the specific antimicrobial mechanism, specific steps must occur to induce bacterial killing[51].

Attraction. Antimicrobial peptides must first be attracted to bacterial surfaces, and one obvious mechanism is electrostatic bonding between anionic or cationic peptides and structures on the bacterial surface. Studies show that peptides like magainin 2 and cecropin A, for example, readily insert into monolayers, large unilamellar vesicles and liposomes that contain acidic phospholipids[50,74]. However, Gram-negative and Gram-positive bacteria are much more complex than model membranes and cationic antimicrobial peptides are likely to first be attracted to the net negative charges that exist on the outer envelope of Gram-negative bacteria — for example, anionic phospholipids and phosphate groups on lipopolysaccharide (LPS) — and to the teichoic acids on the surface of Gram-positive bacteria. Artificial chimeric peptides such as CEME bind to LPS[75] and lipoteichoic acid[76]. The ability of CEME-related peptides to bind to lipoteichoic acid does not correlate with their ability to kill bacteria, indicating that peptides might use this mechanism to contact other targets, such as the cytoplasmic membrane.

Attachment. Once close to the microbial surface, peptides must traverse capsular polysaccharides before they can interact with the outer membrane, which contains LPS in Gram-negative bacteria, and traverse capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in Gram-positive bacteria. This concept is important but is rarely addressed in mechanistic studies. Once peptides

Table 1 | **Membrane and intracellular models of antimicrobial peptide killing and lysis**

Model of antimicrobial activity	Synonym	Examples of peptides
Transmembrane pore-forming mechanisms		
Toroidal pore	Wormhole, disk	Magainin 2[70], protegrin-1[62], melittin[55,81], LL-37[65] and MSI-78[90]
Carpet		Dermaseptin S[85], cecropin[156,157], melittin[158], caerin 1.1[159] and ovispirin[64]
Barrel stave	Helical-bundle model	Alamethicin[61,81]
Modes of intracellular killing		
Flocculation of intracellular contents		Anionic peptides[30]
Alters cytoplasmic membrane septum formation		PR-39[109], PR-26[109], indolicidin[110] and microcin 25[111]
Inhibits cell-wall synthesis		Mersacidin[112]
Binds nucleic acids		Buforin II[113] and tachyplesin[114]
Inhibits nucleic-acid synthesis		Pleurocidin[115], dermaseptin[115], PR-39[73], HNP-1, -2[44] and indolicidin[110]
Inhibits protein synthesis		Pleurocidin[115], dermaseptin [115], PR-39[73], HNP-1, -2[44] and indolicidin[110]
Inhibits enzymatic activity		Histatins[117], pyrrhocoricin, drosocin and apidaecin[118]

have gained access to the cytoplasmic membrane they can interact with lipid bilayers. *In vitro* studies of antimicrobial peptides incubated with single or mixed lipids in membranes or vesicles show that peptides bind in two physically distinct states[77]. At low peptide/lipid ratios, α-helical peptides, β-sheet peptides and θ-defensins adsorb and embed into the lipid head group region in a functionally inactive state (referred to as the surface or S state) that stretches the membrane[72]. The extent of membrane thinning is specific to the peptide and directly proportional to the peptide concentration. In comparison with the peptides magainin 2 (REF. 78), protegrin[79] and alamethicin[80], the membrane-thinning effect of RTD-1 is markedly reduced[41,66].

Peptide insertion and membrane permeability. At low peptide/lipid ratios, peptides are bound parallel to a lipid bilayer[81]. As the peptide/lipid ratio increases, peptides begin to orientate perpendicular to the membrane. At high peptide/lipid ratios, peptide molecules are orientated perpendicularly and insert into the bilayer, forming transmembrane pores (referred to as the I state). The I state peptide/lipid ratio varies with both the peptide and target lipid composition[55], and a number of models have been proposed to explain membrane permeabilization (TABLE 1).

In the 'barrel-stave model' (FIG. 3), peptide helices form a bundle in the membrane with a central lumen, much like a barrel composed of helical peptides as the staves[81,82]. This type of transmembrane pore is unique and is induced by alamethicin. Oriented circular dichroism[55,81], neutron scattering[81] and synchrotron-based X-ray scattering[67] have shown that alamethicin adopts an α-helical configuration, attaches to, aggregates and inserts into oriented bilayers that are hydrated with water vapour. The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore. The alamethicin-induced transmembrane pores can contain 3–11 parallel helical molecules, and the inner and outer diameters have been calculated as ~1.8 nm and ~4.0 nm, respectively[67,68]. The walls of the channel are ~1.1 nm, which is approximately the diameter of the alamethicin helix and is consistent with eight alamethicin monomers arranged according to the barrel-stave model[81,83]. However, changes in bilayer lipid composition can modulate peptide aggregation equilibria and the number of peptides in the aggregate[84].

In the 'carpet model' (FIG. 4), peptides accumulate on the bilayer surface[85]. This model explains the activity of antimicrobial peptides such as ovispirin[64] that orientate parallel ('in-plane') to the membrane surface[61]. Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer in a detergent-like manner, eventually leading to the formation of micelles[86,87]. At a critical threshold concentration, the peptides form toroidal transient holes in the membrane, allowing additional peptides to access the membrane. Finally, the membrane disintegrates and forms micelles after disruption of the bilayer curvature[61,88].

In the 'toroidal-pore model' (FIG. 5), antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups[89]. This type of transmembrane pore is induced by magainins, protegrins and melittin[81,89,90]. In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids[62]. The lipids in these openings then tilt from the lamellar normal and connect the two leaflets of the membrane, forming a continuous bend from the top to the bottom in the fashion of a toroidal hole; the pore is lined by both the peptides and the lipid head groups, which are likely to screen and mask cationic peptide charges[81]. The toroidal model differs

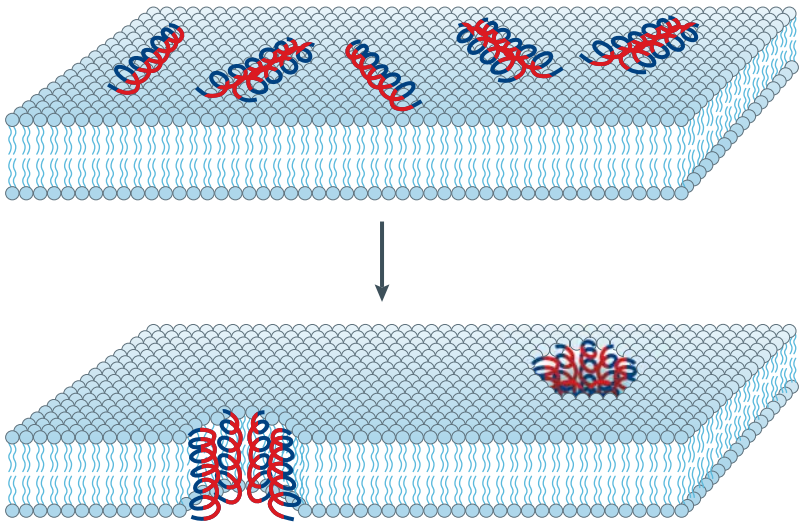

Figure 3 | **The barrel-stave model of antimicrobial-peptide-induced killing.** In this model, the attached peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions align with the lipid core region and the hydrophilic peptide regions form the interior region of the pore. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue. Modified with permission from REF. 88 © (1998) Wiley.

from the barrel-stave model as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer[81]. Otherwise, the presence of several monomers in a toroidal pore would result in a COULOMB ENERGY that is too high for pore formation[81].

Magainin-induced toroidal pores are larger and have a more variable pore size than alamethicin-induced pores[81]. They have an inner diameter of 3.0–5.0 nm and an outer diameter of ~7.0–8.4 nm, and each pore is thought to contain only 4–7 magainin monomers and ~90 lipid molecules [81,91,92].

The mechanisms of defensins are not as well defined[12,13] but they also permeabilize membrane bilayers containing negatively charged phospholipids[12,13,93]. In planar lipid bilayers, HNP-1 and rabbit NP-1 form transmembrane pores when a physiologically relevant negative potential is applied to the membrane side opposite to the defensin-containing diluent[54]. In large unilamellar vesicles, NP-1 creates large, transient defects in phospholipid bilayers[49] and NHP-2 forms 2.5-nm pores[94]. Insect defensin A from *Phormia terramovae* forms oligomeric channels in bacterial membranes, inducing potassium leakage[95], and sapecin from *Sarcophaga peregrina* forms oligomers in phospholipid vesicles[96]. Sapecin interacts with the membrane through basic and hydrophobic residues and then oligomerizes with other sapecin molecules to form pores.

Although descriptions of membrane damage seem to vary, they are likely to be related. It has been suggested that ion channels, transmembrane pores and extensive membrane rupture do not represent three completely different modes of action, but instead are a continuous graduation between them[97]. This concept correlates with the observation that the formation of peptide-induced ultrastructural lesions lags behind the loss of cell viability[44]. Initially, the transmembrane potential and pH gradient are destroyed, the osmotic regulation is affected and respiration is inhibited[92,98–101]. Cecropin A dissipates ion gradients in lipid vesicles at a concentration much lower than that required to release encapsulated calcein, indicating that the bactericidal activity of cecropin A at low concentrations is due to the dissipation of transmembrane electrochemical ion gradients[74]. The treatment of *E. coli* with magainin 2 immediately results in a loss of cytoplasmic potassium and cell death[92], and the treatment of *Micrococcus luteus* with insect defensin A from *P. terramovae* reduces the cytoplasmic potassium concentration, partially depolarizes the inner membrane, reduces the cytoplasmic ATP concentration and inhibits respiration[95].

Models of intracellular killing. Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to the lysis of microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. There is increasing evidence to indicate that antimicrobial peptides have other intracellular targets (FIG. 6). Some early observations revealed that there are alternate sites of antimicrobial peptide activity — for example, Bac7 fragments 1–16, 1–23 and 1–35 did not permeabilize *E. coli* but caused a 2–5 log reduction in the number of organisms[26].

Non-membrane external targets such as autolysins and phospholipases are activated by antimicrobial peptides. In *Staphylococcus simulans*, an autolysin, *N*-acetylmuramoyl-L-alanine amidase, which is inhibited in cell-wall extracts by the presence of lipoteichoic and teichuronic acids, is reactivated by adding the cationic peptide Pep5 (REF. 102), which might explain the lysis of treated cells. Interestingly, in the absence of lipoteichoic acids the activity of partially purified autolysins is also stimulated directly by Pep5. The activity of host-derived secretory phospholipase A_2 for liposomes that contain anionic phospholipids or phosphatidylcholine is markedly enhanced by magainin 2, indolicidin and temporins B and L in 5 μM Ca^{2+} (REF. 103). This synergistic activity, particularly with human lacrimal fluid secretory phospholipase A_2, is likely to be important in the innate response to infection.

Peptides must cross the cytoplasmic membrane and they have developed unique mechanisms to translocate to the cytoplasm. Buforin II, which is a linear, α-helical peptide with a proline hinge, does not permeabilize the cytoplasmic membrane but penetrates it and accumulates in the cytoplasm[35]. The mechanism by which this peptide is translocated was revealed by fusing the proline-hinge region of buforin II with a non-cell-penetrating peptide and the amino-terminal helix of magainin 2 — the hybrid peptides readily penetrated bacterial cytoplasmic membranes and accumulated in the cytoplasm, with concomitant antimicrobial activity[35]. In another study, the helical amphipathicity for 5(6)-carboxyfluoresceinyl-KLALKLALKALKAALKLA-NH_2 was the only essential correlate of cellular uptake[104]. Arginine-rich peptide groups, such as arginine-rich TAT-related peptides, NLS

COULOMB ENERGY
The energy that one stationary, electrically charged substance of small volume exerts on another. For example, in pores formed from numerous cationic peptides, the Coulomb energy would be so high that pore formation would not be possible unless the positive charges are effectively screened when the peptides insert into the membrane containing anionic phospholipids.

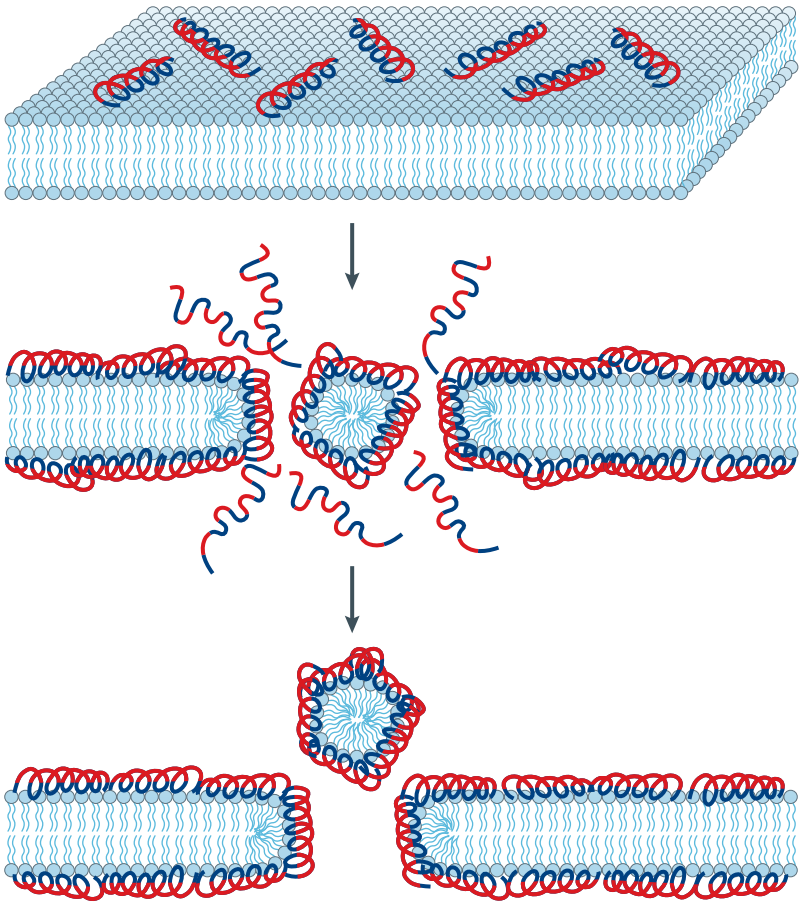

Figure 4 | **The carpet model of antimicrobial-induced killing.** In this model, the peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive layer or carpet. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue. Modified with permission from REF. 88 © (1998) Wiley.

peptides, RNA-binding peptides, DNA-binding peptides and polyarginine and arginine-rich antimicrobial peptides, all readily and efficiently translocate across both cellular and nuclear membranes[105]. In eukaryotic cells, the $TAT_{48–60}$ peptide and Arg_9 are internalized by endocytosis[106], and TAT-fusion proteins are internalized by lipid-raft-dependent macropinocytosis[107]. Apidaecin, a short, proline-rich antibacterial peptide, is translocated by a permease/transporter-mediated mechanism[108].

Once in the cytoplasm, translocated peptides can alter the cytoplasmic membrane septum formation, inhibit cell-wall synthesis, inhibit nucleic-acid synthesis, inhibit protein synthesis or inhibit enzymatic activity (TABLE 1).

PR-39, which is a proline–arginine-rich neutrophil peptide, and its N-terminal 1–26 fragment, PR-26, induce filamentation of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), and indolicidin induces filamentation of *E. coli*[109,110]. Cells exposed to these peptides have an extremely elongated morphology, which indicates that the peptide-treated cells are unable to undergo cell division. This seems to be a common mechanism, as microcin 25, which is a peptide antibiotic exported by *E. coli*, induces long aseptate filaments in other *E. coli*, *Salmonella* and *Shigella* strains at 0.6–2.5 μg ml^{-1} (REF. 111). It is not known whether cell filamentation is due to the blocking of DNA replication or the inhibition of membrane proteins that are involved in septum formation.

Lantibiotics are antimicrobial peptides from Gram-positive bacteria that contain the thioether amino acid lanthionine. The lantibiotic mersacidin inhibits peptidoglycan biosynthesis by interfering with membrane-associated transglycosylation[112]. Mersacidin combines with lipid II, which prevents peptidoglycan precursors, such as undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc (lipid II), from becoming polymeric nascent peptidoglycan.

Buforin II binds to *E. coli* DNA and RNA and alters their electrophoretic mobilities in 1% agarose gels[113], and tachyplesin binds in the DNA minor groove[114]. α-helical peptides (pleurocidin and dermaseptin), proline- and arginine-rich peptides (PR-39 and indolicidin) and defensins (HNP-1) block (^{3}H)thymidine, (^{3}H)uridine and (^{3}H)leucine uptake in *E. coli*, showing that they inhibit DNA, RNA and protein synthesis[44,73,110,115]. At their minimal inhibitory concentrations, pleurocidin and dermaseptin both inhibit nucleic acid and protein synthesis without damaging the *E. coli* cytoplasmic membrane[115]. PR-39 (25 μM) stops protein synthesis and induces degradation of some proteins that are required for DNA replication[73]. HNP-1 and -2 (50 μg ml^{-1}) can reduce DNA, RNA and protein synthesis, and HNP-1 also inhibits the synthesis of periplasmic β-galactosidase[44]. Indolicidin (100 μg ml^{-1}) completely inhibits DNA and RNA synthesis in *E. coli* but does not have any effect on protein synthesis[110]. At concentrations of 150 and 200 μg ml^{-1}, protein synthesis is markedly inhibited.

Histatins bind to a receptor on the fungal cell membrane, enter the cytoplasm and induce the non-lytic loss of ATP from actively respiring cells[116]. Their action can also disrupt the cell cycle and lead to the generation of reactive oxygen species[117].

Short, proline-rich antibacterial peptides also have several different effects. Pyrrhocoricin, drosocin and apidaecin bind specifically to DnaK, a 70-kDa heat-shock protein[118], and nonspecifically to GroEL, a 60-kDa bacterial chaperone[118]. Pyrrhocoricin reduces the ATPase activity of recombinant DnaK[119] and pyrrhocoricin and drosocin alter the refolding of misfolded proteins indicating that drosocin and pyrrhocoricin binding prevents the frequent opening and closing of the multihelical lid over the peptide-binding pocket of DnaK, permanently closes the cavity and inhibits chaperone-assisted protein folding[119].

Mechanisms of bacterial resistance

Microorganisms use a number of resistance strategies to circumvent antimicrobial peptide killing and these mechanisms are important for the concepts presented in this review (BOX 3). These bacterial strategies counter mechanisms of antimicrobial peptide attachment, peptide insertion and membrane permeability.

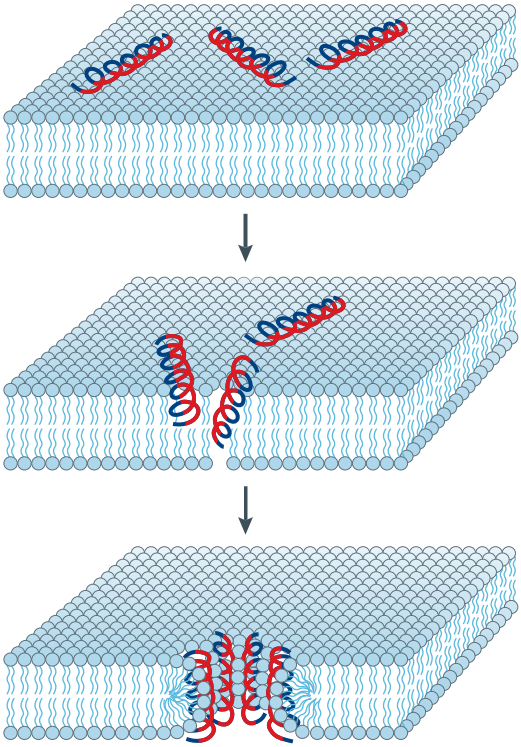

Figure 5 | **The toroidal model of antimicrobial peptide-induced killing.** In this model the attached peptides aggregate and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue. Modified with permission from REF. 162 © (2004) American Physical Society.

In *Staphylococcus aureus*, products of the *dlt* operon, which includes the genes *dltA*, *dltB*, *dltC* and *dltD*, reduce the net negative surface charges by transporting D-alanine from the cytoplasm to the surface teichoic acid[120]. The teichoic acid backbone is highly charged by deprotonized phosphate groups, and esterification with D-alanine causes a reduction of the net negative charge by adding basic amino groups. Inactivation of the *dlt* operon confers sensitivity to defensins, protegrins and other antimicrobial peptides. *S. aureus* also modifies its anionic membranes with L-lysine through MprF, which encodes a lysylphosphatidylglycerol (LPG) synthetase. LPG is a basic phospholipid formed by the transfer of L-lysine from lysyl-tRNA to phosphatidylglycerol[121,122]. This increases the positive net charge and is likely to repulse host defence peptides. *mprF* mutants are susceptible to killing by neutrophil defensins, indicating a central role for defensin resistance in the pathogenicity of *S. aureus*.

Gram-negative bacteria reduce their susceptibility to antimicrobial peptides by hindering peptide attachment to the outer membrane, reducing net negative surface charges by altering the Lipid A moiety of the LPS or by reducing the fluidity of the outer membrane by increasing the number of hydrophobic interactions between the increased number of Lipid A acyl tails. Capsule polysaccharide mediates resistance of *Klebsiella pneumoniae* to HNP-1, HBD1, lactoferrin, protamine sulphate and polymyxin B[123]. The capsule polysaccharide limits the interaction of the peptides with their membrane targets, and *K. pneumoniae* strain 52K10, an acapsular mutant, is more susceptible to peptide-mediated killing than *K. pneumoniae* strain 52145, the capsulated clinical isolate.

Aminoarabinose can be added to Lipid A phosphate groups, which reduces the electrostatic interaction between cationic antimicrobial peptides and the negatively charged phosphate bound in ester linkage to the fourth carbon of glucosamine II[124]. 2-hydroxymyristate and palmitate can also be added to Lipid A, which is likely to reduce the fluidity of the outer membrane due to increased hydrophobic interactions between the increased number of Lipid A acyl tails. The increased hydrophobic interaction retards or abolishes peptide insertion and pore formation. These alterations are activated by the two-component regulatory system PhoP–PhoQ in *S. typhimurium*[125] and *P. aeruginosa*[126]. This regulon comprises a sensor kinase, PhoQ, and a transcriptional activator, PhoP[127], which is expressed in response to environmental signals including changes in extracellular magnesium or calcium concentrations, pH or other signals after infection or phagocytosis[128]. In *S. typhimurium* this system can simultaneously activate or repress more than 40 different genes, known as PhoP-activated genes (*pag/pqa*) and PhoP-repressed genes (*prg/pqr*)[129]. PhoP–PhoQ also regulates the PmrA–PmrB two-component regulatory system, which in turn controls transcription of the genes *pmrE/ugd* and the *pmrF* operon. PmrA–PmrB regulates the resistance of *P. aeruginosa* to polymyxin B and cationic antimicrobial peptides in low-Mg^{2+} conditions and induces putative LPS modifications[126]. Inactivation of genes in the PhoP–PhoQ regulon and assessment of the sensitivity of mutants to antimicrobial peptides have identified both the genes and the respective modifications of Lipid A that are involved in antimicrobial peptide resistance[127,129].

In some Gram-negative bacteria such as *Yersinia enterocolitica*, alterations in outer membrane proteins increase resistance to antimicrobial peptides. Resistance to killing by antimicrobial peptides is correlated with the presence of a 70-kb plasmid, designated pYVe, which encodes *Y. enterocolitica* adhesin A (YadA), *Y. enterocolitica* lipoprotein A (YlpA) and the production of Yop proteins[130].

Antimicrobial resistance is also associated with the ability to either transport antimicrobial peptides into the cell by the ATP-binding cassette transporter[131,132] or to export antimicrobial peptides by the resistance-nodulation cell-division efflux pump[133,134]. Both mechanisms require energy and active transport of peptide for antimicrobial resistance.

The antimicrobial-peptide-resistance mechanisms described above often do not account for all the resistance seen in Gram-negative bacteria[128] and increasing evidence indicates that proteolytic enzymes might also

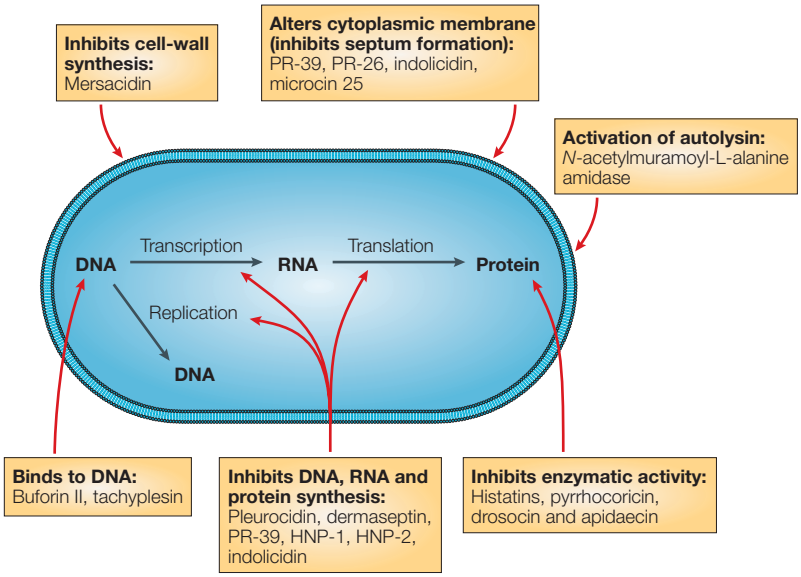

Figure 6 | **Mode of action for intracellular antimicrobial peptide activity.** In this figure *Escherichia coli* is shown as the target microorganism

be involved[132,135–137]. For example, LL-37 is cleaved and inactivated by an *S. aureus* metalloproteinase named aureolysin[138] — *S. aureus* strains that produce significant amounts of aureolysin are less susceptible to the antimicrobial fragment LL-17-37 than are strains that do not express aureolysin. Whether proteolysis of antimicrobial peptides is a bacterial mechanism of resistance *in vivo* has yet to be proven.

The basis of peptide activity and specificity

There is extensive information about the characteristics and traits of effective antimicrobial peptides, the locations at which they are produced and their ranges of activities[26,28,33,36,139]. Although many relationships between peptide structure and antibacterial activity have been described[26,33,140], little is known about the molecular basis of the marked differences in peptide activity and specificity. For example, the differences in the susceptibility of a single microorganism to a panel of antimicrobial peptides indicate that the size, the sequence, the degree of structuring (for example, helical content), the charge, the overall hydrophobicity, the amphipathicity and the respective widths of the hydrophobic and hydrophilic faces of the helix (BOX 2) are all important[26,141]. Additional traits are also important and a distinct carboxyl terminus stereospecific γ-loop sequence was identified in defensins and found to correlate with antimicrobial activity[142]. Similar loops were found in previously unrecognized peptides such as the sweetener brazzein and the scorpion neurotoxin charybdotoxin, which were found to have antimicrobial activity against bacteria and fungi. Altering any of the above parameters, including the hydrophobic moment and the angle subtended by charged residues, can modify antimicrobial and haemolytic activity of peptides[98]. As noted by Tossi *et al.*, many of these parameters are closely related and altering one parameter can result in significant changes in the others[33]. This indicates that there are limits to the amount of 'tweaking' that can be done to develop the ideal antimicrobial peptide.

Box 3 | **Characteristics of bacteria that are resistant to antimicrobial peptides**

Alteration of net surface charges

Staphylococcus aureus transports D-alanine from the cytoplasm to the surface teichoic acid to reduce the net negative charge by introducing basic amino groups. *S. aureus* also modifies its anionic membranes via MprF with L-lysine, increasing the positive net charge. Both of these mechanisms are likely to repulse host defence peptides, which indicates a central role for antimicrobial peptide resistance in the pathogenicity of *S. aureus*.

Capsule polysaccharide of *Klebsiella pneumoniae* limits the interaction of antimicrobial peptides with membrane targets and acapsular mutants are more susceptible to peptide-mediated killing[123].

In *Salmonella* species, the phosphate group linked to glucosamine I of Lipid A is substituted by a phosphorylethanolamine residue with a free amino group and aminoarabinose is added to the negatively charged phosphate bound in ester linkage to the carbon-4 of glucosamine II of Lipid A.

Alterations in Lipid A

Salmonella species reduce the fluidity of their outer membrane by increasing hydrophobic interactions between an increased number of Lipid A acyl tails by adding myristate to Lipid A with 2-hydroxymyristate and forming hepta-acylated Lipid A by adding palmitate. The increased hydrophobic moment is though to retard or abolish antimicrobial peptide insertion and pore formation.

Changes in membrane proteins

In some Gram-negative bacteria, for example *Yersinia enterocolitica*, alteration in the production of outer membrane proteins correlates with resistance to killing by antimicrobial peptides.

Role of transporters

ATP-binding cassette transporters import antimicrobial peptides[131,132] and the resistance-nodulation cell-division efflux pump exports antimicrobial peptides[133,134]. Both transporters have been associated with antimicrobial peptide resistance.

Proteolytic enzymes

Bacteria produce proteolytic enzymes, which may degrade antimicrobial peptides leading to their resistance[132,135-137]. For example, LL-37 is cleaved and inactivated by a *S. aureus* metalloproteinase named aureolysin[138].

Alternately, the differences in the susceptibility of a panel of microorganisms to a single peptide indicate that the composition of the microbial surface and cytoplasmic membrane is equally important[10,140]. A certain amount of innate antimicrobial resistance is related to the structure and composition of the LPS molecule in the outer membrane of Gram-negative bacteria and the phospholipids in the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria. Membrane lipid composition is important[143] and bacteria with cytoplasmic membranes that are enriched in acidic phospholipids are more susceptible to antimicrobial peptides[92]. Finally, effective definitions of antimicrobial peptide activity and specificity should take into consideration the physiological conditions *in vivo*. This includes the concentrations of antimicrobial peptides at the sites of infection, the role of synergistic substances that might be present in tissues and fluids (for example, the presence of lysozyme, other antimicrobial peptides and proteins, and the absence of divalent cations), the role of inhibiting substances that might be present (for example, physiological concentrations of salts and serous proteins) and the unusual characteristics of bacteria replicating *in vivo*, particularly those in biofilms[144].

In summary, differences among peptides and differences among bacterial surfaces and cytoplasmic membranes are just a few of the variables that determine the extent of antimicrobial-peptide-induced bacterial killing. So, designing antimicrobial peptides on the basis of their ability to induce transmembrane pores alone might be of limited benefit in combating a small subset of microorganisms. However, recognition that antimicrobial peptides have targets other than membranes, such as lactoferrin, which blocks biofilm development by opportunistic pathogens[145], or that antimicrobial peptides have synergistic effects with other host innate immune molecules such as human lacrimal fluid secretory phospholipase A_2[103] will facilitate the development, design and synthesis of more efficient, broad-spectrum therapeutic antimicrobial peptides.

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Competing interests statement

The author declares no competing financial interests.

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